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14. ABSTRACT

The overall goal of this project is to probe the CTL – tumor cell interaction by generating scFv probes that are able to recognize the HLA-A*0201-HER-2/neu369-377 peptide complex. In the 12 month period covered by this report, I aimed to improve the affinity of HLA-A*0201-HER-2/neu369-377 peptide complex-specific scFv 2.3.5, which was isolated during the first 12 month period of this project. Utilizing three rounds of site-directed mutagenesis in the VL CDR3 and VL CDR1 of scFv 2.3.5, I have generated and isolated a scFv clone, named 2.3.5-58-53, which exhibited markedly higher affinity for the HLA-A*0201-HER-2/neu369-377 peptide complex than the parental scFv 2.3.5. I will utilize scFv 2.3.5-58-53 to analyze the levels of HLA-A*0201:HER-2/neu369-377 complexes expressed by breast carcinoma cell lines and breast carcinoma lesions, in order to determine if there are correlations between the levels of antigen processing machinery components, HLA class I antigens, β2-microglobulin, and/or HER-2/neu on the levels of HLA-A*0201:HER-2/neu369-377 complex on breast carcinoma cells.

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INTRODUCTION

This Annual Summary is the second annual report of DOD Predoctoral Fellowship Award BC030039, entitled “CTL – Tumor Cell Interaction: The generation of molecular probes capable of monitoring the HLA-A*0201-HER-2/neu peptide complex”. It is intended to communicate the research progress, with particular emphasis on key research accomplishments and reportable outcomes, related to this award mechanism in the 12-month period spanning from February 2005 through February 2006.

The low affinity of the HLA-A*0201:HER-2/$neu_{369-377}$ complex-specific scFv 2.3.5, which was isolated in the first 12-month funding period, represented a limitation to its utility in the detection of HLA-A*0201:HER-2/$neu_{369-377}$ complexes on breast carcinoma cells. To enhance the affinity of this reagent, we utilized molecular biology approaches to introduce site-specific amino acid changes in the complementarity-determining regions (CDRs) of scFv 2.3.5, with the aim of isolating a clone with higher affinity for the HLA-A*0201:HER-2/$neu_{369-377}$ complex. We performed three rounds of mutagenesis: (i) Alanine-scanning mutagenesis of V_L CDR3; (ii) Alanine-scanning mutagenesis of V_L CDR1; and (iii) Site-directed random mutagenesis of V_L CDR1. We obtained a scFv clone, named 2.3.5-58-53, which has been mutagenized in both its V_L CDR3 (M89T, Q90A, L94V) and V_L CDR1 (L27cA, H27dA, S27eP) regions. scFv 2.3.5-58-53 exhibited markedly higher affinity for the HLA-A*0201:HER-2/$neu_{369-377}$ complex than the parental scFv 2.3.5.

We will utilize scFv 2.3.5-58-53 to analyze the levels of HLA-A*0201:HER-2/$neu_{369-377}$ complexes that are expressed by a panel of breast carcinoma cell lines and breast carcinoma lesions. The data obtained from this analysis will allow us to determine if there are correlations between the levels of antigen processing machinery components, HLA class I antigens, β2-microglobulin, and/or HER-2/neu on the levels of HLA-A*0201:HER-2/$neu_{369-377}$ complex on breast carcinoma cells.

I. Enhancement of the affinity of scFv 2.3.5 by site-directed mutagenesis of CDR sequences

The HLA-A*0201:HER-2/neu$_{369-377}$ complex-specific scFv 2.3.5 was isolated in the first 12-month funding period of this grant. However, a limitation that we encountered was the low affinity of this reagent, which limited its utility for detecting complexes on HLA-A2^{+}HER-2/neu^{+} breast carcinoma cells.

To address this issue, during the previous 12-month funding period of this grant we utilized a series of molecular biology approaches to introduce site-specific mutations in the complementarity-determining region (CDR) sequences of scFv 2.3.5. The wild-type nucleotide and amino acid sequence of scFv 2.3.5 is shown in **Fig. 1**.

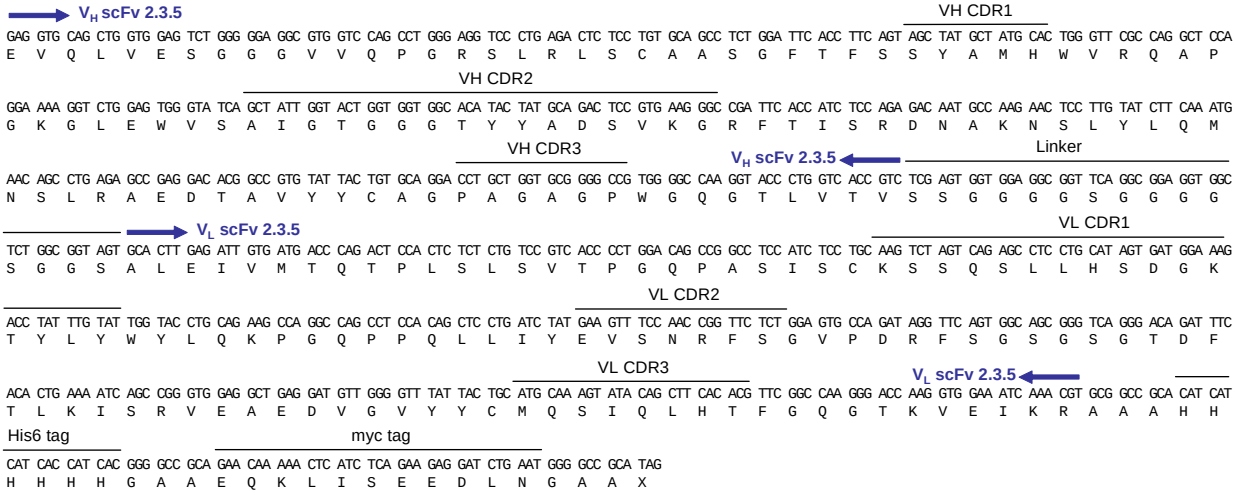

Fig. 1. Wild-type nucleotide and amino acid sequence of scFv 2.3.5.

II. Alanine-scanning mutagenesis of V_L CDR3 sequence of scFv 2.3.5

We focused on the CDRs associated with the light chain (V_L CDR1, V_L CDR2, and V_L CDR3), since these were determined to be more similar to the corresponding human germline sequences. Among these, we focused first on V_L CDR3, since the CDR3 sequence is considered to be the most critical for the antibody:antigen interaction.

The V_L CDR3 nucleotide sequence of scFv 2.3.5 has 3 hot spots, which are defined as nucleotide sequences (i) A/G-G-C/T-A/T or (ii) A-G-C/T (**Fig. 2**). Based on the positions of the known hot spots in V_L CDR3, we performed a round of alanine-scanning mutagenesis (**1, 2**) targeting residues 89-94. Briefly, the DNA sequence encoding wild-type scFv 2.3.5 was subjected to PCR utilizing a degenerate forward primer and an overlapping reverse primer (**Table 1**), in order to introduce a series of mutations at positions 89-94 (**Fig. 3**).

Position	89	90	91	92	93	94	95	96
Codon	ATG	CAA	AGT	ATA	CAG	CTT	CAC	ACG
Amino acid	M	Q	S	I	Q	L	H	T

Hot spot sequence: A/G-G-C/T-A/T and AGY (Y=C or T)

Fig. 2. V_L CDR3 nucleotide and amino acid sequence of scFv 2.3.5.

These DNA sequences were then introduced into phage particles to construct a secondary phage-display scFv library. The phage particles from this library were amplified via standard methodologies that we have utilized previously, and were panned against T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide. The scFv clones obtained following panning were tested for their reactivity to T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide by flow cytometry. As a specificity control, the scFv clones were also tested against T2 cells pulsed with MART-1$_{26-35}$ peptide. One higher affinity clone, named 2.3.5-58 (M89T, Q90A, L94V) was selected for further analysis.

Alanine scanning position	Target sequence	Primer sequence
V_L CDR3 residues 89-94	ATG CAA AGT ATA CAG CTT	Forward primer: 48mer GGGGTTTATTACTGCRYGSMAKCCRYTSMASYTCACACGTTCGGCCAA Reverse primer: 39mer GCAGTAATAAACCCCAACATCCTCAGCCTC

Table 1. Alanine scanning mutagenesis targeting residues 89-94 in V_L CDR3 of scFv 2.3.5. The highlighted portion of the forward primer (which spans from codons 84-99) corresponds to the nucleotides that encode residues 89-94 in V_L CDR3. The reverse primer spans from codons 88-79. K=T/G; M=A/C; R=A/G; S=C/G; Y=C/T.

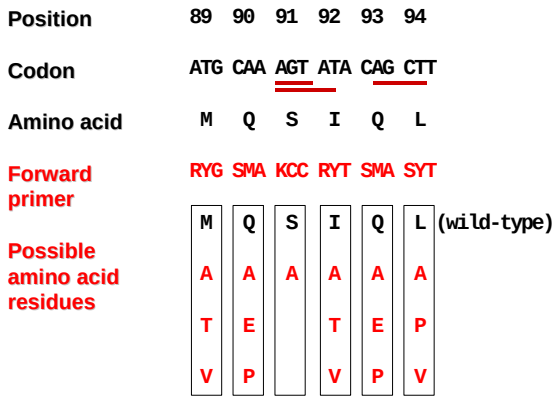

Fig. 3. Possible amino acid residue changes in V_L CDR3 of scFv 2.3.5 following alanine scanning mutagenesis.

III. Alanine-scanning mutagenesis of V_L CDR1 sequence of scFv 2.3.5-58

Next, we determined if mutagenizing the V_L CDR1 sequence of scFv 2.3.5-58 could further improve the specific reactivity of this scFv to the HLA-A*0201:HER-2/neu$_{369-377}$ complex. The V_L CDR1 nucleotide sequence of scFv 2.3.5-58 (which is identical to the V_L CDR1 sequence of the parental scFv 2.3.5) has three hot spots (**Fig. 4**). Based on the positions of the known hot spots in V_L CDR1, we performed a round of alanine-scanning mutagenesis targeting residues 26-27e. Briefly, the DNA sequence encoding scFv 2.3.5-58 was subjected to PCR

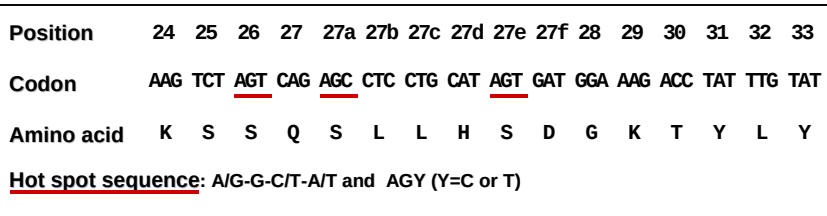

Fig. 4. V_L CDR1 nucleotide and amino acid sequence of scFv 2.3.5-58.

Alanine scanning position	Target sequence	Primer sequence
V_L CDR1 residues 26-27e	AGT CAG AGC CTC CTG CAT AGT	Forward primer: 50mer CCATCTCCTGCAAGTCTKCCSMAKCCSYTSYTSMTKCCGATGGAAAGACC Reverse primer: 29mer AGACTTGCAGGAGATGGAGGCCGGCTGTC

Table 2. Alanine scanning mutagenesis targeting residues 26-27e in V_L CDR1 of scFv 2.3.5-58. The highlighted portion of the forward primer (which spans from codons 20-30) corresponds to the nucleotides that encode residues 26-27e in V_L CDR1. The reverse primer spans from codons 25-16. K=T/G; M=A/C; S=C/G; Y=C/T.

utilizing a degenerate forward primer and an overlapping reverse primer (**Table 2**), in order to introduce a series of mutations at positions 26-27e (**Fig. 5**). These DNA sequences were then introduced into phage particles to construct a tertiary phage-display scFv library. The phage particles from this library were amplified, and were then panned against T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide. The scFv clones obtained following panning were tested for their reactivity to T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide by flow cytometry. As a specificity control, the scFv clones were also tested against T2 cells pulsed with MART-1$_{26-35}$ peptide. All of the clones that were selected for sequencing following this screening step had the wild-type sequence at positions 26-27b. Therefore, a further round of affinity maturation was utilized to introduce mutations at positions 27c-27e of the V_L CDR1 of scFv 2.3.5-58.

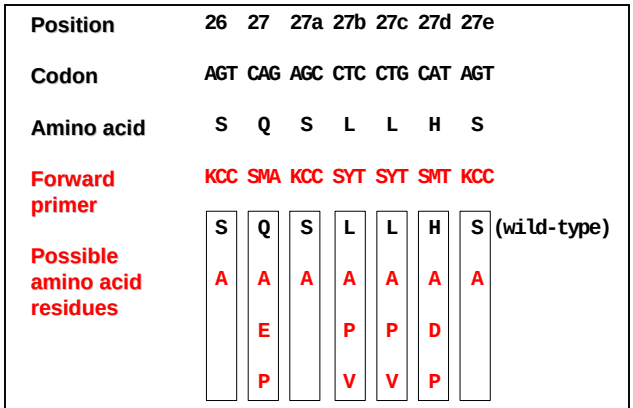

Fig. 5. Possible amino acid residue changes in V_L CDR1 of scFv 2.3.5-58 following alanine scanning mutagenesis.

IV. Site-directed random mutagenesis of V_L CDR1 sequence of scFv 2.3.5-58

To introduce random residues at positions 27c-27e of the V_L CDR1 of scFv 2.3.5-58, the DNA sequence encoding scFv 2.3.5-58 was subjected to PCR utilizing a degenerate forward primer and an overlapping reverse primer (**Table 3**). Unlike alanine-scanning mutagenesis approaches utilized previously, the forward primer utilized for random mutagenesis will be able to introduce random amino acids at any of the targeted positions. As in **Sections II and III**, these DNA sequences were then introduced into phage particles to construct an additional phage-display scFv library. The phage particles from this library were amplified, and were then panned against T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide. The scFv clones obtained following panning were tested for their reactivity to T2 lymphoid cells pulsed with HER-2/neu$_{369-377}$ peptide by flow cytometry. As a specificity control, the scFv clones were also tested against T2 cells pulsed with MART-1$_{26-35}$ peptide. A higher affinity clone, named 2.3.5-58-53 (L27cA, H27dA, S27eP), was isolated. As shown in **Fig. 6**, this variant of scFv 2.3.5, which has mutations both in its V_L CDR3 and V_L CDR1 regions compared with the

Site-directed mutagenesis position	Target sequence	Primer sequence
V_L CDR1 residues 27c-27e	CTG CAT AGT	Forward primer: 42mer AAGTCTAGTCAGAGCCTCNNSNNSNNSGATGGAAAGACCTAT Reverse primer: 33mer GAGGCTCTGACTAGACTTGCAGGAGATGGAGGC

Table 3. Site-directed random mutagenesis targeting residues 27c-27e in V_L CDR1 of scFv 2.3.5-58. The highlighted portion of the forward primer (which spans from codons 24-31) corresponds to the nucleotides that encode residues 27c-27e in V_L CDR1. The reverse primer spans from codons 27b-19. N=A/C/T/G; S=C/G.

original scFv, exhibits higher affinity to T2 cells pulsed with HER-2/neu$_{369-377}$ peptide than either the original scFv 2.3.5 or the scFv 2.3.5-58 mutant isolated in **Section II**.

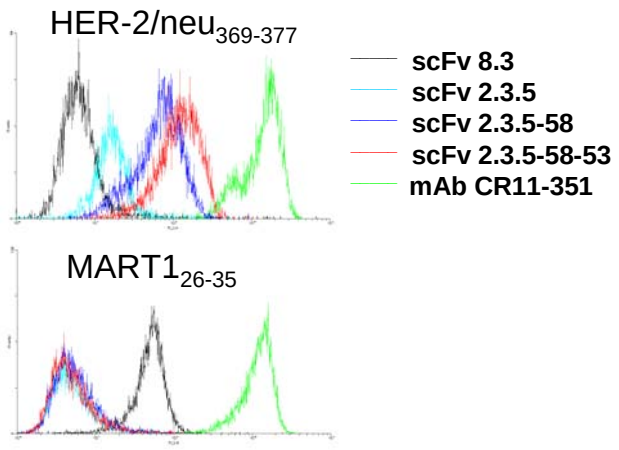

Fig. 6. Reactivity of mutagenized scFv clones with peptide-pulsed T2 cells. Mutagenized scFv clones 2.3.5-58 and 2.3.5-58-53 were compared with the parental scFv 2.3.5 in their reactivity with T2 cells pulsed with HER-2/neu$_{369-377}$ peptide (20 µg/ml) (top panel). The HLA-A*0201:MART126-35 complex-specific scFv 8.3 and the HLA-A2,A24,A28-specific mAb CR11-351 were utilized as negative and positive controls, respectively. T2 cells pulsed with MART126-35 peptide (50 µg/ml) (bottom panel) were utilized as a specificity control.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the level of antigen presentation machinery (APM) components and HLA class I antigen expression in human breast carcinoma cell lines by FACS analysis, utilizing panels of mAb specific for APM components, HLA class I antigen, and β2m that have been developed in the Ferrone laboratory (continuation of work performed in the first 12-month funding period).

- Performed site-directed mutagenesis of V_L CDR3 and V_L CDR1 in HLA-A*0201:HER-2/$neu_{369-377}$ complex-specific scFv 2.3.5 to increase its affinity.

- Isolated one HLA-A*0201:HER-2/$neu_{369-377}$ complex-specific scFv clone (2.3.5-58-53) following site-directed mutagenesis, and demonstrated that scFv 2.3.5-58-53 exhibits higher affinity for HER-2/$neu_{369-377}$ peptide-pulsed T2 cells than the parental scFv 2.3.5.

REPORTABLE OUTCOMES

1. Ogino T, Miyokawa N, Ko E, Ferrone S. HLA class I antigen abnormalities in cancer: molecular mechanism, clinical significance and negative impact for T cell-based immunotherapy. *Manuscript in preparation*.

CONCLUSIONS

In the course of the last 12 months, I have successfully carried out a series of mutagenesis experiments to enhance the affinity of the HLA-A*0201:HER-2/neu$_{369-377}$ complex-specific scFv 2.3.5. A higher affinity variant of the parental scFv, which has been named scFv 2.3.5-58-53, was isolated following three rounds of mutagenesis. scFv 2.3.5-58-53 will be utilized in future studies to assess the levels of HLA-A*0201:HER-2/neu$_{369-377}$ complex on breast carcinoma cells, and to determine if there are correlations between the levels of APM components, HLA class I antigens, β2m, and/or HER-2/neu on the levels of HLA-A*0201:HER-2/neu$_{369-377}$ complexes.

In conclusion, the research activities supported by this DOD Predoctoral Fellowship Award have served as an ideal training vehicle for a wide range of laboratory techniques, including (i) the analysis of protein antigens with mAb, by utilizing Western blotting, immunohistochemistry, and flow cytometry technologies; (ii) the generation of recombinant proteins in a bacterial system by DNA manipulation with molecular biology techniques; and (iii) the screening, isolation, and analysis of HLA-A*0201:HER-2/neu$_{369-377}$ complex-specific scFv clones.

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2. Morrison KL, Weiss GA. Combinatorial alanine-scanning. Curr Opin Chem Biol 2001; 5:302-307.